Preparation of New Hydrophilic Poly-vinylpyridine Beads and Their Application to Immobilization of Urease

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Synopsis

Macroporous hydrophilic poly-vinylpyridine beads were prepared by suspension copolymerization of 4- (or 2-)vinylpyridine and hydrophilic cross-linking agents, such as ethylene glycol dimethacrylate (1EG) and tetraethylene glycol dimethacrylate (4EG), and their physical properties were studied. Immobilized enzymes were obtained by adsorption of urease on vinylpyridine polymers and commercially available hydrophobic polymers. The poly-4-vinylpyridine resin crosslinked with 4EG (degree of crosslinkage, 25%) showed higher water regain, larger average pore radius, and the highest adsorption of urease. Leakage of urease from immobilized urease was scarcely observed in the buffer solution used. The hydrophilicity and hydrophobicity of support affected markedly the enzymatic reaction of the immobilized enzyme. As one of the applications of the polymer-supported urease, blood urea nitrogen in human sera was determined.

INTRODUCTION

Immobilized enzyme has been developed for overcoming drawbacks of free enzyme, such as reusability, ease of handling, and heat instability. Many articles have reported on immobilized enzymes prepared by covalent bonding.[1] But chemical modifications of enzyme for covalent bonding are accompanied by a lowering of enzymatic activity, inactivation, and shift of optimum pH of enzyme. Though the preparation of immobilized enzymes by adsorption has the disadvantage of leakage, some advantages of this method include: free movement of the molecules on the matrices, little change in the enzyme's property, and simple preparation without specified reagents. Activated carbon, inorganic materials, and ion-exchange resins mainly have been used as adsorbents for enzyme.[2] However, there are only a few examples relating to properties of resins as the matrices for adsorption of enzyme.[3]

Vinylpyridine resins have been applied as ion-exchange resins, chelating resins, and packing materials for gas chromatography and high-performance liquid chromatography.[4-7] As the resins are crosslinked with divinylbenzene (DVB), they are low in hydrophilicity in some degree, therefore, buffer solutions of neutral to alkaline reagents insufficiently permeate into the vinylpyridine resins. Polymers containing pyridine moiety have been converted into polyaldehyde by the reaction with cyanogen bromide and the polyaldehydes formed were applied to matrices for immobilization of proteins by covalent bonding.[8]

In this work, several novel macroporous hydrophilic vinylpyridine resins were prepared by suspension copolymerization method with vinylpyridine and

Journal of Applied Polymer Science, Vol. 32 4931–4938 (1986)
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hydrophilic cross-linking reagents such as ethylene glycol dimethacrylate (1EG) and tetraethylene glycol dimethacrylate (4EG). The applicability of the resins as supports for immobilized enzyme was studied.

EXPERIMENTAL

Materials

All reagents were of commercial reagent grade. 4- (or 2-)vinylpyridine (Wako Pure Chemical Ind. Ltd.) was purified by distillation before use. Divinylbenzene (50–55%, Wako Pure Chemical Ind. Ltd.) and ethylene glycol dimethacrylate (Tokyo Kasei Kogyo Co. Ltd.) were washed with 5% sodium hydroxide solution to remove stabilizer. Tetraethylene glycol dimethacrylate (Shin Nakamura Chemical Co. Ltd.) and urease (E.C.3.5.15., 71 units/mg, from Jack Bean, Worthington Diagnostic System Corp.) were used without further purification. Amberlite XAD-2 and XAD-8 were obtained from Japan Organo Co., Ltd. and were purified by washing with organic solvents and dried. Buffer solution used in enzymatic studies was 0.1 *M* phosphate buffer containing 5 m*M* EDTA.

Preparation of Vinylpyridine (VP) Resins and Their Physical Properties. Polymerizations were carried out under a nitrogen atmosphere. Organic phase consisted of a mixture of 4-vinylpyridine (4VP) or 2-vinylpyridine (2VP), cross-linking reagents, diluent, and benzoyl peroxide (1% of the monomers) dispersed in an aqueous phase containing 1.0 or 0.5% hydroxyethyl cellulose, 10% sodium chloride, and 0.4% sodium hydroxide and stirred at room temperature. The temperature of the mixture was raised to 70°C after 1 h of stirring. After stirring for 4 h at 70°C, the resulting resins were washed with hot water until they were free from the adhering stabilizer. Beads of particle sizes between 60 and 100 mesh were collected and used in adsorption studies of urease. Water regain, weight swelling ratio, and surface properties were determined in the usual way.

Adsorption of Urease on Resins. A urease solution of 50 mL (5 mg urease/100 mL phosphate buffer (0.1 *M*, pH 7.0)) was added to the 1 g of resin and the mixture was incubated overnight at 4°C. Then the resin was filtered off and washed thoroughly with the buffer solution until the filtrate showed no enzymatic activity. The buffer solution adhered to the resin was blotted off with filter paper and the supported urease was stored in a refrigerator at 4°C.

Enzymatic Activity of the Supported Urease. Amount of urease adsorbed on resins: 200 mg of the supported urease was added to 1 mL of the buffer solution (pH 7.0) and then 10 mL of the buffer solution containing 5% of urea was added. The time course of the enzymatic reaction was studied at 38°C. The concentration of ammonia formed at each definite time in aqueous phase was determined by a urease–indophenol method[9] and the amount of enzyme adsorbed on the resins was determined by comparing the initial reaction rate of the supported enzyme with the reaction rate obtained from the standard solution of free urease solution.

RESULTS AND DISCUSSION

Conditions for preparation of the vinylpyridine beads and their properties were shown in Tables I, II, and III. For the preparation of vinyl/pyridine

resins, 4VP or 2VP was used as vinylpyridine monomer and 1EG or 4EG was applied as a hydrophilic crosslinker. Vinylpyridine resin crosslinked with DVB, which is a hydrophobic crosslinker, was also prepared for comparison. In the preparation of the resins, organic solvents (toluene, tert-buthanol, and iso-octane) were added to the monomers to obtain macroporus structure. The resins crosslinked with hydrophilic 4EG were lyophilized because they showed aggregation when air dried. Weight swelling ratios of resins are shown in Table IV. As expected, 4VP-4EG-25 had very high swelling ratios. The resin crosslinked with 4EG showed the highest water regain among the resins tested and this suggests the resin had very high hydrophilicity.

Enzymatic activity of urease adsorbed on various kinds of vinylpyridine resins are summarized in Table V. As shown, the quantity of urease adsorbed on the resins is decreases with increasing crosslinkage. The amounts of urease adsorbed on 4VP-4EG, whose degree of crosslinkage is different, are shown in Figure 1. Pyridyl moiety in the resins decreased with increased crosslinkage and highly crosslinked resins become more rigid. In the adsorption of urease

TABLE I
Conditions for Preparation of Vinylpyridine Resins

Resin	Monomer (mL) Vinyl pyridine	Cross-linking reagent	Diluent (mL) T B I	Dispersion medium	Benzoyl peroxide (g)
4VP-1EG-25	4VP(60)	1EG(20)	(24)(16)(0)	0.5%,[a] 600 mL	0.8
4VP-1EG-50	4VP(40)	1EG(40)	(32)(8)(0)	0.5%, 600 mL	0.8
4VP-4EG-25	4VP(60)	4EG(20)	(64)(16)(0)	1.0%, 600 mL	0.8
4VP-4EG-50	4VP(40)	4EG(40)	(64)(16)(0)	0.5%, 800 mL	0.8
4VP-4EG-75	4VP(20)	4EG(60)	(72)(0)(0)	0.5%, 600 mL	0.8
4VP-DVB-25	4VP(40)	DVB(40)	(64)(0)(0)	1.0%, 600 mL	0.8
2VP-4EG-25	2VP(60)	4EG(20)	(0)(0)(36)	0.5%, 600 mL	0.8

[a] Concentration of hydroxyethyl cellulose. 4VP: 4-vinylpyridine; 2VP: 2-vinylpyridine; 1EG: ethylene glycol dimethacrylate; 4EG: tetraethylene glycol dimethacrylate; DVB: divinylbenzene; T: toluene; B: tert-buthanol; I: iso-octane.

TABLE II
Properties of Resins

Resin	Nitrogen content (mEq/g)	Specific surface area[a] (m^2/g)	Pore volume[b] (cm^3/g)	Average pore radius (nm)
4VP-1EG-25	6.68	3.52	0.18	16.4
4VP-1EG-50	4.51	33.40	0.26	15.4
4VP-4EG-25	7.08	3.40	1.38	632.2
4VP-4EG-50	4.35	3.52	0.76	113.9
4VP-4EG-75	2.12	0	0.04	12.0
4VP-DVB-25	4.76	92.9	0.36	13.8
2VP-4EG-25	6.66	0.86	1.00	453.0
XAD-2[c]	—	300	0.69	4.5
XAD-8[c]	—	140	0.82	11.8

[a] Determined by BET method.
[b] Determined by mercury porosimetry.
[c] Data taken from Ref. 10.

TABLE III
Water Regains of Vinylpyridine Resins

Resin	Water regain (g water/g resin)
4VP-1EG-25	0.91
4VP-1EG-50	0.72
4VP-4EG-25	2.48
4VP-4EG-50	1.82
4VP-4EG-75	1.30
4VP-DVB-25	1.07
2VP-4EG-25	1.91

TABLE IV
Weight Swelling Ratios (SWR) of Vinylpyridine Resins

Solvent	4VP-1EG -25	4VP-1EG -50	4VP-4EG -25	4VP-4EG -50	4VP-4EG -75	4VP-DVB -25	2VP-4EG -25
Water	0.00	0.00	1.41	0.61	0.00	0.00	0.00
Buffer	0.00	0.00	1.48	0.65	0.00	0.00	0.00
Methyl alcohol	0.40	0.00	5.62	1.41	0.61	0.40	1.01
Ethyl alcohol	0.79	0.40	3.36	1.19	0.60	0.60	1.00
n-Propyl alcohol	1.20	0.71	1.15	1.74	1.16	0.71	1.06
n-Butyl alcohol	0.80	0.55	0.96	0.81	0.50	0.75	0.10
n-Amyl alcohol	0.65	0.30	0.40	0.38	0.15	0.80	0.00
Benzyl alcohol	1.53	0.40	8.80	3.19	2.42	0.80	3.84

Resin was swelled at 25°C for 24 h; buffer: 0.1 *M* phosphate containing 5 m*M* EDTA, pH 7.0; SWR = $(V_2 - V_1)/W$, V_1: volume (mL) of resin before swelling, V_2: volume (mL) of resin after swelling, W: weight (g) of resin.

TABLE V
Enzymatic Activity of Immobilized Urease and Amounts of Adsorbed Urease

Resin	Enzymatic activity of immobilized urease at 25°C[a] (unit/g resin)	Urease adsorbed[b] (μg urease/g resin)
4VP-1EG-25	4.37	61.5
4VP-1EG-50	0.24	3.4
4VP-4EG-25	23.86	336.0
4VP-4EG-50	5.59	78.7
4VP-4EG-75	2.56	36.0
4VP-DVB-25	4.54	64.0
2VP-4EG-25	12.64	178.0
XAD-2	7.31	103.0
XAD-8	0.97	13.6

[a]Specific activity: 71 units/mg urease.
[b]Calculated values based on the enzymatic activity.

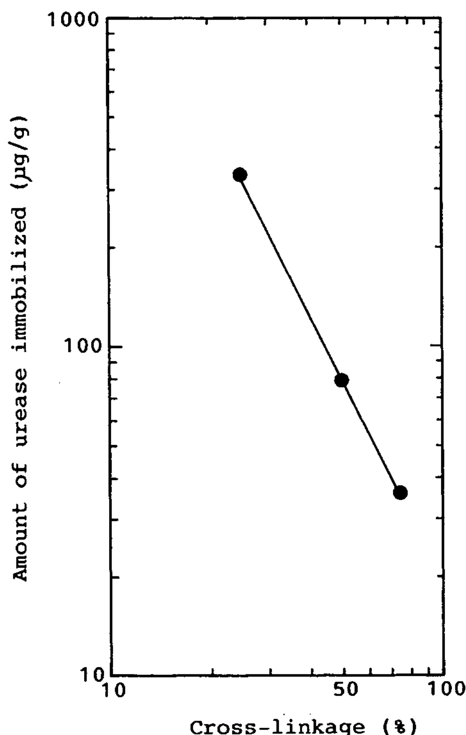

Fig. 1. Relationship between crosslinkage of resins and amount of urease immobilized. Resin: 4VP-4EG copolymers.

on the resins, hydrophobic interaction between urease and the resins seems to play an important role. With regard to enzymatic activity, the following factors should be taken into account. The enzyme substrate is easily approached by the enzyme on the support and the enzymatic reaction product can be removed readily from the reaction sites.

The 4VP-4EG-25 and XAD-2 (copolymer of styrene and DVB), which is one of the commercially available hydrophobic synthetic adsorbents, were applied to the support for the urease and the effect of the support for the enzymatic activity was determined (Fig. 2). In this experiment, the substrate is added in large excess compared to the amount of urease on the support, the enzymatic reaction should ideally proceed linearly with incubation time. In the case of 4VP-4EG-25-urease, the concentration of the reaction product increased linearly with incubation time. However, in the case of XAD-2-urease, the reaction is saturated in about five minutes. This indicates that hydrophilicity of the support plays a very important role in the enzymatic reaction.

Langmuir plot of adsorption of urease on the 4VP-4EG-25 is shown in Figure 3. The adsorption of urease on the resin is presumed to be completed in a single molecular phase. However, the amount of urease on the resin is not proportional to the surface area of the resin. This indicates that the surface area measured by using nitrogen does not directly reflect the amount of urease adsorbed on the resins, because the molecular weight of urease is relatively large (483,000) and its structure is revolving ellipsoidal, having a long axis

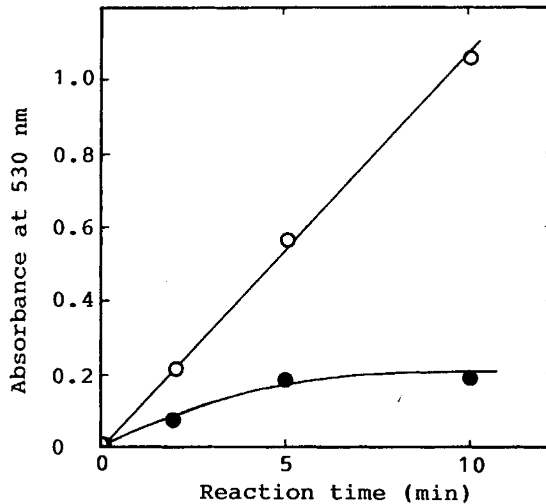

Fig. 2. Effect of support on enzymatic reaction. (○) 4VP-4EG-25-urease, 100 mg; (●) XAD-2-urease, 100 mg; substrate: 5% urea/buffer solution, 5 mL; reaction temperature: 38°C; buffer: 0.1 *M* phosphate buffer containing 5 m*M* EDTA, pH 7.0.

(26.5 nm) and a short axis (6.5 nm).[11] The amount of urease adsorbed on the resin seems to depend on the pore size of the resin rather than the surface area.

The relationship between the amount of urease adsorbed and pore size of the resins (25% crosslinkage) is shown in Figure 4. The amount of urease adsorbed shows an exponential increase with respect to the average pore radius of the resins, and this behavior suggests that the pore radius gives a substantial contribution to the adsorption of urease on the resins.

As mentioned above, 4VP-4EG-25 was found to have outstanding characteristics for the support of urease. The supported urease (4VP-4EG-25-urease) was used for the further studies.

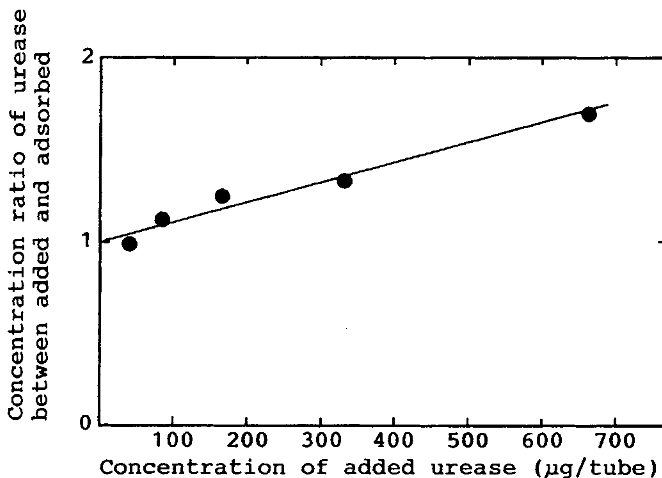

Fig. 3. Langmuir's adsorption isotherm of urease on 4VP-4EG-25. Resin: 4VP-4EG-25, 500 mg; temperature: 27°C.

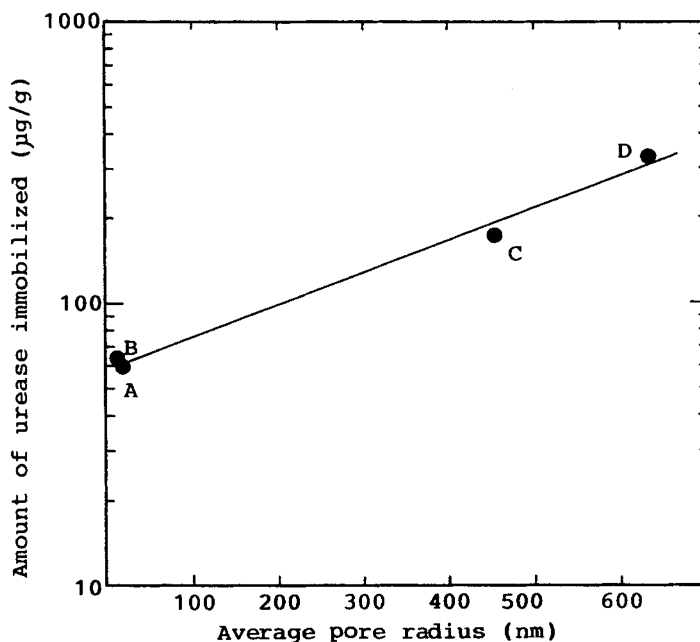

Fig. 4. Relationship between average pore radius of resins and amount of urease immobilized. (A): 4VP-1EG-25; (B): 4VP-DVB-25; (C): 2VP-4EG-25; (D): 4VP-4EG-25.

The apparent Michaelis constant was obtained from a Lineweaver-Burk plot.[12] The apparent Michaelis constants for 4VP-4EG-25-urease and free urease were 56.0 m*M* and 10.6 m*M*, respectively. The apparent Michaelis constant of the supported enzyme was about six times as large as that of the free enzyme, but the value is similar to those of other kinds of supported enzymes.[13] The temperature of the maximum enzymatic reaction was near 60°C and the optimum pH of the supported urease is similar to that of free enzyme. When the supported enzyme was kept at 4°C for one month, the activity was not changed, however when held at the same temperature for 2 months, the activity was 80% of the initial activity.

The leakage of the immobilized urease from the supported urease was hardly detectable in the phosphate buffer (0.1 *M*, pH 7.0), therefore the supported urease can be assumed to be useful in a wide range of applications.

TABLE VI
Determination of Blood Urea Nitrogen (BUN) by Urease-Indophenol Method

	BUN (mg N/dL)	
Sample	Immobilized urease	Urease solution
A	13.9 ± 0.3	13.5 ± 0.2
B	16.1 ± 0.5	14.9 ± 0.5
C	10.0 ± 0.8	8.7 ± 0.6
D	13.1 ± 0.2	12.8 ± 0.5

Sample: normal human sera, 100 μL for immobilized urease, 10 μL for urease solution, $n = 5$; immobilized urease: 100 mg; urease solution: 5 mg-urease/50 mL-buffer solution, 500 μL.

Recycling of the supported urease was also investigated. The enzymatic activity did not change in more than 10 recycling times.

As a possible clinical application of 4VP-4EG-25-urease, the concentration of blood urea nitrogen (BUN) in normal human sera was determined (Table VI). The data obtained by the supported urease showed almost reasonable values (normal value for adults: 8-20 mg N/dL) compared with standard methods using free enzyme.[14]

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Received September 24, 1985
Accepted March 8, 1986